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DISTRIBUTION OF BEE POLLEN GRANULES ACCORDING TO VIBRATION SPECTROSCOPIC MARKERS

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Annotation. *The present work is devoted to the vibration spectroscopic analysis of the distribution of bee pollen granules of four botanical species: Trifolium repens, Papaver somniferum, Brassica napus and Phacelia tanacetifolia. The suitability of FTIR spectroscopy in the near (NIR) and mid (MIR) infrared regions as well as FT Raman spectroscopy for the analysis of bee pollen granules and their classification according to botanical specie was evaluated.*

The spectroscopic data obtained were statistically processed using principal component analysis (PCA). Each of these methods proved to be suitable for bee pollen classification on the basis of spectral differences. FT Raman spectra confirmed that the presence of carotenes significantly affected the colour of poppy bee pollen.

Key words: spectroscopic *analysis, bee pollen, Trifolium repens, Papaver somniferum, Brassica napus, Phacelia tanacetifolia.*

Bee pollen is an interesting and promising product from beekeeping. It is a mixture of flower pollen and nectar with bee excreta. This bee product was characterised as the source of free amino acids, proteins, fats, fatty acids, mono- and polysaccharides, antioxidants, vitamins and pigments [1, 2]. For high nutritional value and balanced ratio of healthy substances it is suitable for therapeutic applications [3]. Chemical and biochemical composition of bee pollen depends mainly on its botanical origin, but also on the time of harvesting, soil and climatic conditions. Identification and classification is required for the appropriate and effective use and quality evaluation of this beekeeping product.

Bee pollens of various origins have specific botanic and chemical composition, so it is important to identify them and discriminate from each other. Bee pollen granules of unifloral botanical origin commonly have uniform chemical composition, while those of heterofloral origin have variable composition depending of the ratio between the botanical sources. Fractionalisation of bee pollen permits to separate specific chemical components. Chromatographic methods (HPLC, GC) are useful for their identification in the fractions, and some of these compounds (fatty acids, flavonoids, etc.) are markers of botanical origin [4, 5].

However, these methods often need destructive and time consuming sample preparation, and it is necessary to establish specific method for each kind of chemical compounds. By contrast, vibration spectroscopic methods (FTIR, Raman, and NIR) are non-destructive, universal and need no invasive sample preparation. Thus vibration spectroscopy excludes destructive factors like chemical and thermal degradation, migration of components during extraction and purification etc. FTIR and Raman spectroscopy have been applied to structural analysis and discrimination of bee pollens [6–9].

Aim and tasks. The aim of this work is evaluation of in analysis of distribution and homogeneity bee pollen granules in defined unifloral bee pollen material by the use of vibration spectroscopic (FTIR, Raman) and multivariate statistical methods. Following task will be solved: (a) random selection of bee pollen granules from bee pollen material, (b) recording of FT MIR ATR, diffuse reflektance FT NIR and FT Raman spectra of each granule, (c) IR/Raman spectral band assignment and search of spectroscopic markers of chemical components, (d) statistical evaluation of the obtained spectroscopic data.

Materials and methods. Bee pollen samples originated from four botanical species (*Trifolium repens, Papaver somniferum, Brassica napus* and *Phacelia tanacetifolia*) were used for the analyses (Table 1). One hundred randomly selected pollen granules of each botanical species were randomly chosen for recording of the FTIR (MIR) ATR and diffuse reflectance FT NIR spectra. Similarly, 8 groups defined as 1–3 specifically coloured fractions of four species (10 granules in each group) were collected for recording of the FT Raman spectra.

1. Sample description

Botanic origin	Number of granules	
	FT MIR ATR FT NIR	FT Raman
Trifolium repens	100	10 (brown), 10 (yellow)
Papaver somniferum	100	10 (brown), 10 (yellowish brown), 10 (yellow)
Brassica napus	100	10 (yellow), 10 (greenish)
Phacelia tanacetifolia	100	10 (violet)

FT MIR ATR spectra (range 650–4000 cm^{-1}, 64 scans, resolution 2 cm^{-1}) were recorded on FTIR spectrometer Nicolet 6700 (Thermo Scientific, USA) using smart MIRacle holder and Omnic 7.0 software. Diffuse reflectance FT NIR spectra (range 10000–4000 cm^{-1}, resolution 2 cm^{-1}, 64 scans) were recorded on the same spectrometer using smart NIR UpDRIFT holder. FT Raman spectra (range 150–4000 cm^{-1}, 1054 scans, resolution 4.0 cm^{-1}) were recorded on FT Raman spectrometer Equinox 55/S (Bruker, USA) with Nd:YAG laser (λ_{ex}=1064 nm, power 250 mW), silicon beam splitter and Ge detector cooling with liquid N_2. All the spectra were exported to Origin 6.0 (Microcal Origin, USA) software for further processing (smoothing, baseline correction) and preparation of the graphs. Spectra were exported in table to

Statistica 9.0 (Statsoft, USA) software for statistical evaluation. Principal component analysis (PCA) of the spectra was made based on covariation and using TQ Analyst 8.5.21 (Microcal Origin, USA) software.

Results and discussion. FT MIR ATR spectra (3800–650 cm^{-1}) of randomly chosen bee pollen granules from each supposedly unifloral samples are demonstrated in Fig. 1. Spectral differences represent non-similarities in composition and ratio between main constituents, i.e. proteins, sugars, fats, aromatics etc. Fig. 2 represents medians of these sets of spectra, and band assignments are summarised in Table 2 [9]. According to the spectra, granules from *Phacelia tanacetifolia* contained more sugars (1200–950 cm^{-1}, CO and CC stretching), proteins (1653 and 1549 cm^{-1}, amide vibrations) and phenolics (1516 cm^{-1}, C=C stretching) [10]. By contrast, granules from *Trifolium repens* contained more fats (2925, 2855 and 1739 cm^{-1}, CH_2 and C=O stretching), granules from *Papaver somniferum* contained more organic acids (1710 cm^{-1}, C=O stretching).

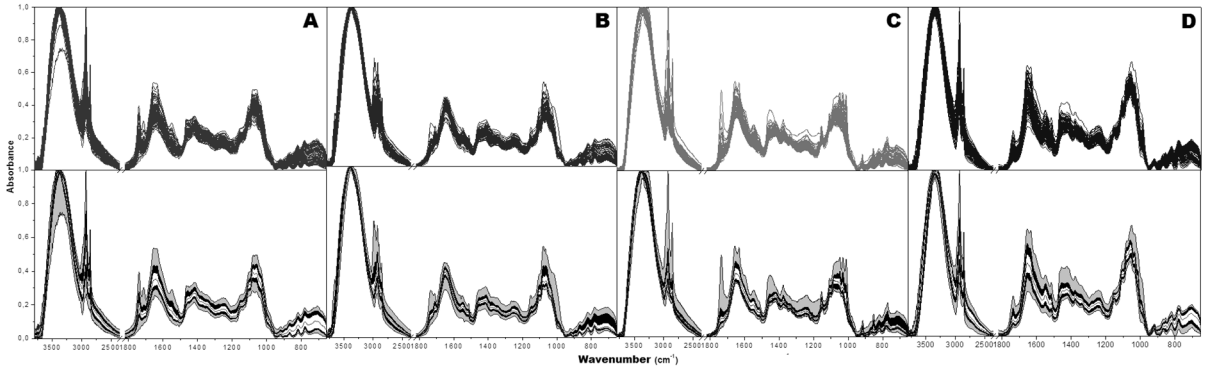

Fig. 1. FT MIR ATR spectra (top) of bee pollen granules (4 sets per 100) and statistical evaluation of the spectra (bottom): *Trifolium repens* (A)*, Papaver somniferum* (B)*, Brassica napus* (C) and *Phacelia tanacetifolia* (D).

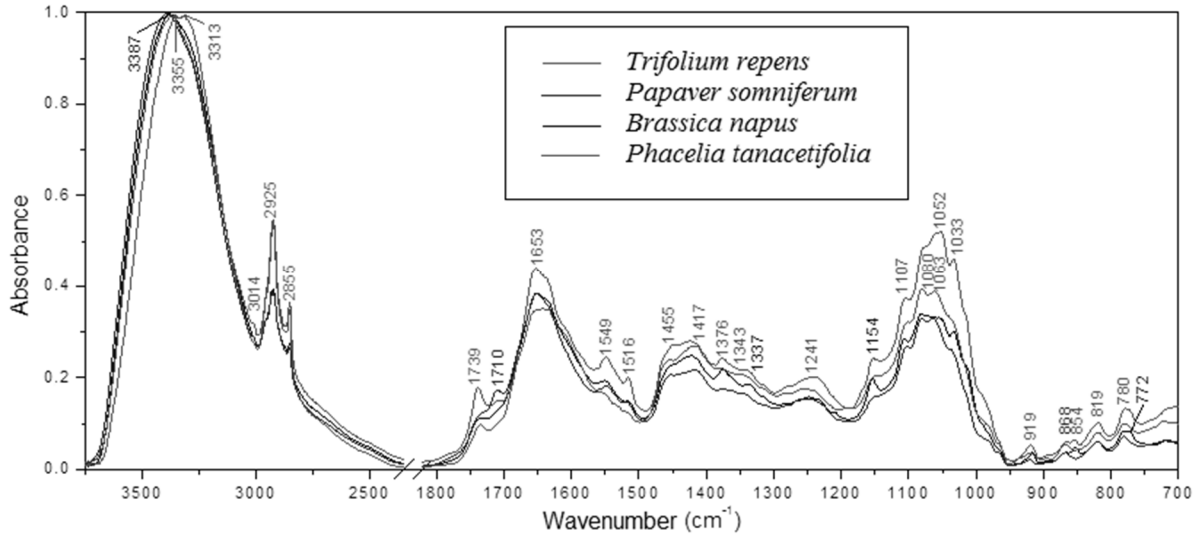

Fig. 2. FT MIR ATR median spectra of bee pollen granules (4 sets per 100).

However, there was a significant diversity in intensities of some bands for granules that is evident from comparing of extremal values in graphs (Fig. 1). All

spectra demonstrate several bands in the region of 921–780 cm^{-1} assigned to amorphous fructose, the main sugar component of nectar [11]. The narrower region (1832–664 cm^{-1}) of FT MIR ATR spectra was chosen for PCA. Loading graph (Fig. 3, top) shows curves of three main components in this region, and 3D component score graph (Fig. 3, bottom) demonstrate different space location of dots corresponding to specific samples. However, the regions of each sample are partially overlapped by the others. There are several outliers in these groups that could be explained by specific composition of some granules (unknown components or another botanic origin).

2. IR band assignment for bee pollen granules of four botanic species

T. repens	*P. somniferum*	*B. napus*	*P. tanacetifolia*	Vibration	Assignment
3383br	3391br	3387br	3355 3313	ν(OH)	water, sugars
3014				ν(=CH)	unsaturated FA
2925	2928	2925	2925	$\nu_{as}(CH_2)$	lipids, proteins, sugars
2855	2855	2855	2855	$\nu_{s}(CH_2)$	
1739		1739	1735	ν(C=O)	fats
1707	1710			ν(C=O)	organic acids
	1650	1650	1653	amid I	proteins
1642				$\delta(H_2O)$	water
1633				ν(C=O)	aromatics
	1548	1549	1549	amid II	proteins
	1517		1516	ν(C=C)	aromatics
1453	1452		1455	$\delta_{as}(CH_3)$	proteins, lipids
1417	1415	1422	1424	δ(COH), δ(CCH)	sugars
	1375	1376	1376	$\delta_{s}(CH_3)$	proteins, lipids
		1337	1343	amid III	proteins
1279			1278		
1256	1246	1249	1241	ν(COC), δ(OH)	esters, sugars
1148		1154	1152		Fru, sugars
1102	1102	1108	1107		
1080	1081	1080	1080	ν(CO)(CC), δ(COH)	sugars
1063	1065	1064	1052		
		1034	1033		
921	921	917	919		
887	868		865		
		853	854		Fru
819	820	820	819	skeletal	
780	781	781	780		
		772			
	719				sugars, proteins, aromatics

Diffuse reflektance FT NIR spectra (1000–4000 cm^{-1}) of randomly chosen bee pollen granules from each supposedly unifloral samples are demonstrated in Fig. 4. Like in the previous case, spectral differences clarify variability in chemical composition of granules. Fig. 5 represents medians of these sets of spectra, and band assignments are summarised in Table 3. Band assignment in NIR region is very complicated because of combination and overtone bands overlapping [9].

The spectra are sensitive to fat to sugar ratio, probably to protein contribution as well. The narrower region (5960–5830 cm^{-1}) of FT NIR spectra was chosen for PCA. Loading graph (Fig. 6, left) shows curves of three main components PC3, PC4 and PC5 in this region, and 3D component score graph (Fig. 6, right) demonstrate that the dots (with some exceptions) create several more or less diffused clusters, which were less overlapped than those of FT MIR ATR spectra. Comparing these clusters, it is evident that bee pollen from *Phacelia tanacetifolia* is the most homogeneous, while bee pollen from *Brassica napus* contained some inclusions from other botanic species.

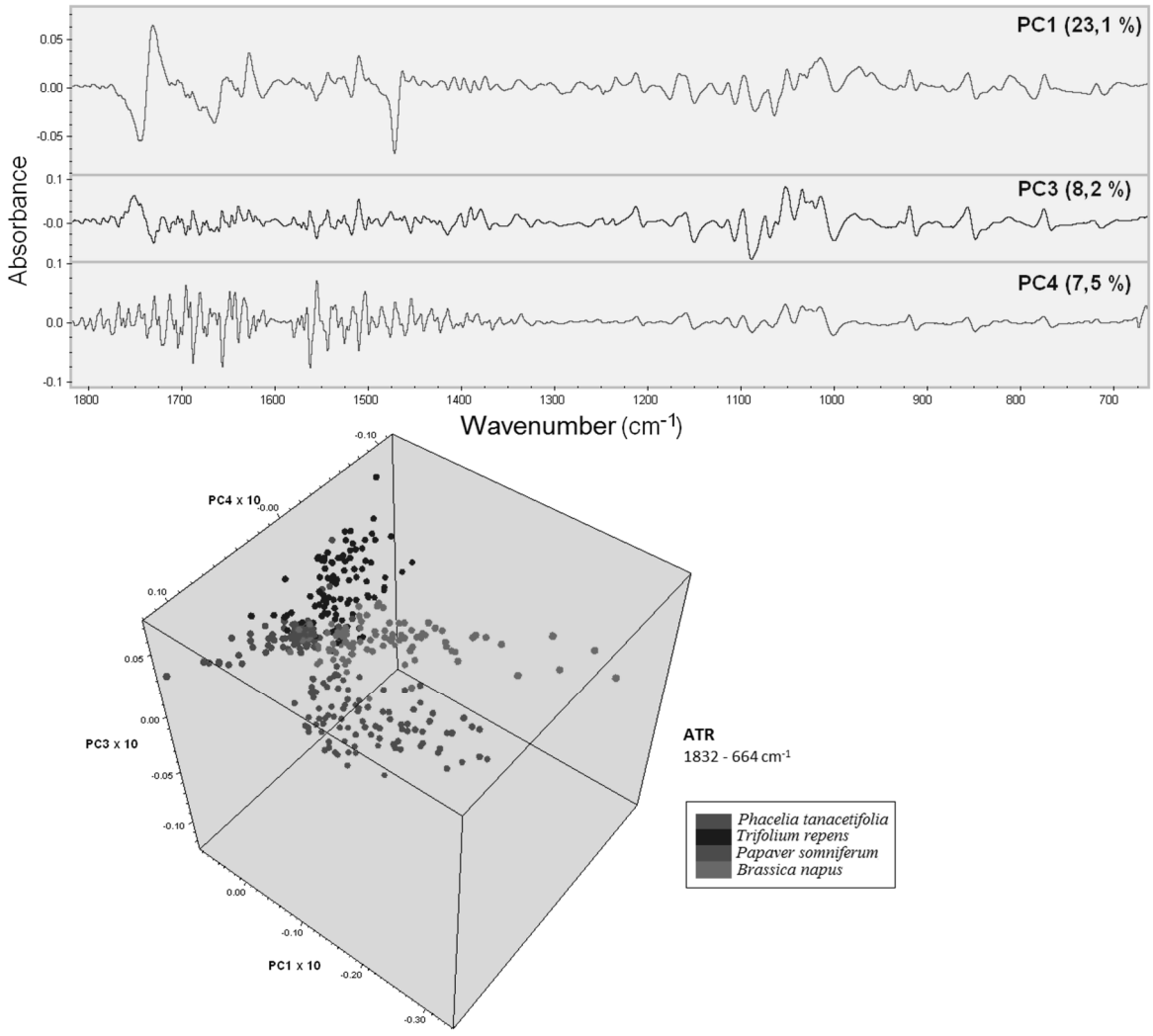

Fig. 3. Loading and 3D component score graphs for FT MIR ATR spectra of bee pollen granules (4 sets per 100)

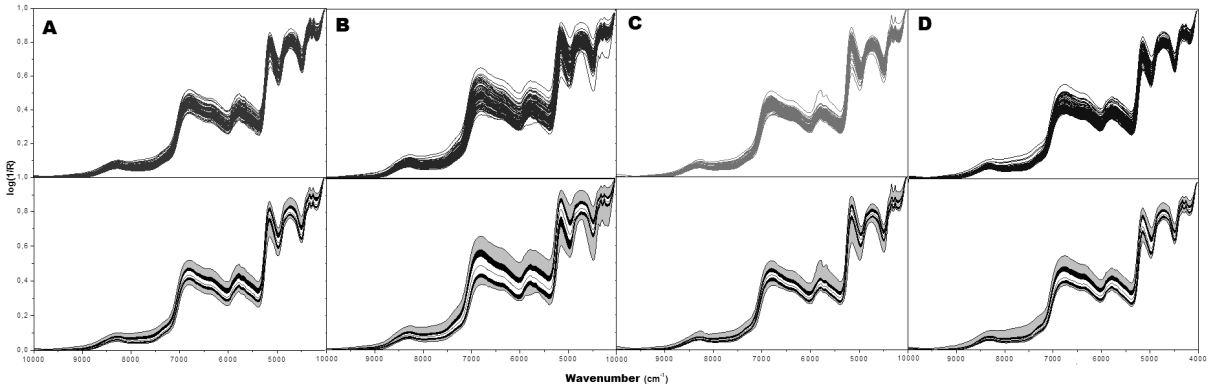

Fig. 4. Diffuse reflectance FT NIR spectra (top) of bee pollen granules (4 sets per 100) and statistical evaluation of the spectra (bottom): *Trifolium repens* (A)*, Papaver somniferum* (B)*, Brassica napus* (C) and *Phacelia tanacetifolia* (D)

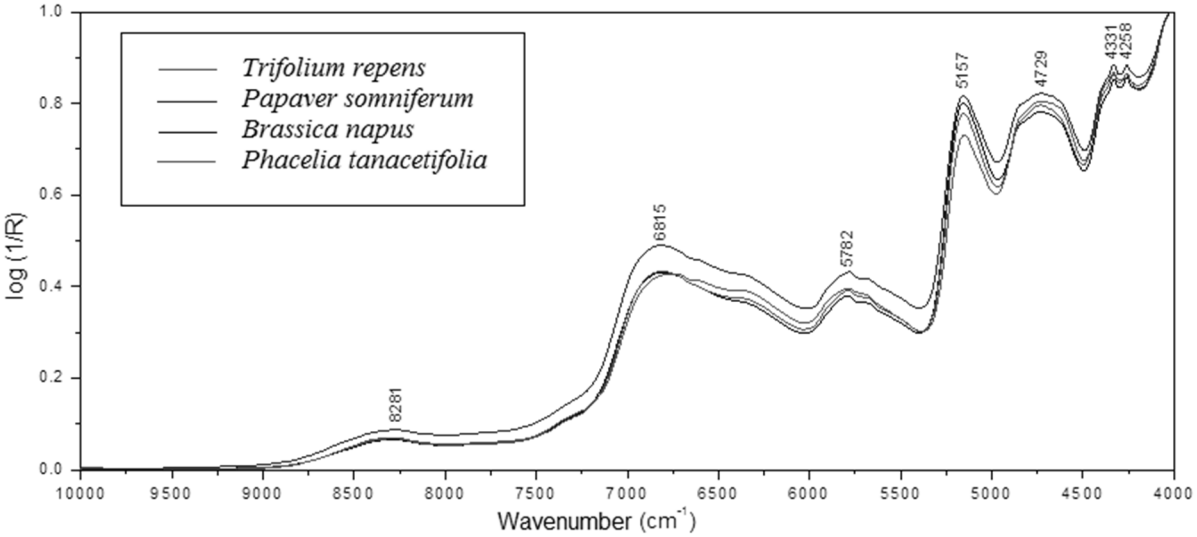

Fig. 5. Diffuse reflectance FT NIR spectra (top) of bee pollen granules (4 sets per 100)

3. Summary table assignment NIR bands of four botanical species

T. repens	*P. somniferum*	*B. napus*	*P. tanacetifolia*	Vibration	Assignment
8281	8281	8281	8281	$3\nu_{as}(CH_2)$	fats
6815	6815	6815	6815	$2\nu(OH)$	water, sugars
5782	5782	5782	5782	$2\nu_{as}(CH_2)$	fats
5157	5157	5157	5157	$2\nu(C=O)$	fats, organic acids
4729	4729	4729	4729	$\nu(OH) + \delta(H_2O)$	water
4331	4331	4331	4331	$\nu_{as}(CH_2) + \delta(CH_2)$	fats
4258	4258	4258	4258	$\nu_{s}(CH_2) + \delta(CH_2)$	

FT Raman spectra (1750–350 cm^{-1}) of randomly chosen bee pollen granules from each supposedly unifloral samples (1–3 fractions of the same colour per each sample) are demonstrated in Fig. 7. Spectral differences represent non-similarities in composition and ratio between main constituents and pigments, i.e. proteins, sugars, fats, aromatics and carotenes. Band assignments are summarised in Table 4.

4. Raman band assignments for bee pollen granules

Wavenumber (cm^{-1})	Vibration	Assignment
1728	ν(C=O)	lipids, organic acids
1654	amide I	proteins
1605	$\nu_{as}(COO^-)$	amino acids
1525	ν(C=C)	β–carotene
1514	ν(C=C)	polyfenoly
1454	$\delta(CH_2)$	lipids, Fru
1440	$\delta(CH_2)$, $\delta_{as}(CH_3)$	
1362	$\delta_s(CH_3)$	proteins
1340	amide III	
1308		
1263	ν(COC), δ(COH)	lipids, Fru
1173	ν(COC)	sugars
1155	$C\text{-}CH_3$	β–carotene
1128		
1080	ν(CC), ν(CO), ν(CN)	proteins, lipids, sugars
1065		
1005	skeletal	β–carotene, Phe
980	ν(CC), ν(CO), ν(CN)	proteins, lipids, sugars
918		
866		
820		
710	skeletal	Fru
629		
519		
420		

The carotene bands at 1520, 1155 a 1005 cm^{-1} demonstrate maximal variability [12]; the last one is overlapped by the protein band near 1004 cm^{-1} (ring breezing of Phe). These bands were intense only for brown granules of pollen from *Papaver somniferum* (Obr. 8), so these is a tight dependence between carotene level and colour.

Next intense bands at 1654, 1605 a 1440 cm^{-1} were assigned to proteins, aromatics and lipids; several bands at 1454, 1263, 1075, 916, 868, 821, 707, 630, 519 and 421 cm^{-1} are typical for amorphous fructose [13].

Two spectral regions were chosen for PCA of FT Raman spectra and their 1st derivatives: (a) 1700–1400 cm^{-1} (C=O and C=C stretching in proteins, carotenes, lipids and aromatics) and 1190–980 cm^{-1} (CC and CO stretching in sugars, lipids and proteins).Two sets of principal components were used (PC1, PC2 and PC6 for spectra; PC1, PC2 and PC3 for 1st derivatives). These

components demonstrated maximal discrimination of granules according to their colour and botanic origin. Loading graph demonstrates PC1–PC6 curves at 1700–980 cm^{-1}; 3D graphs of component score for spectra and 1st derivatives demonstrate discrimination of the clusters. As in case of FTIR, the set of granules from *Phacelia tanacetifolia* showed maximal homogeneity.

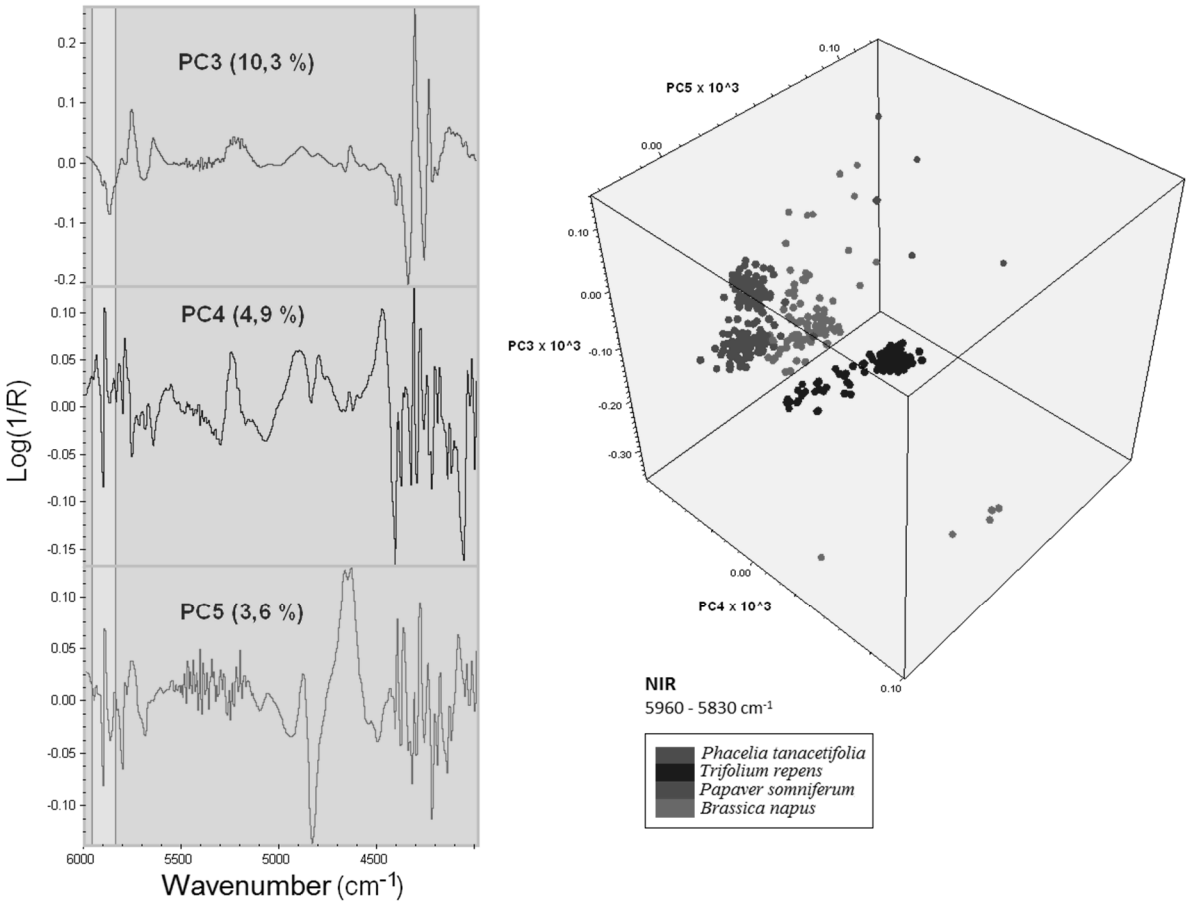

Fig. 6. Loading and 3D component score graphs for diffuse reflectance FT NIR spectra of bee pollen granules (4 sets per 100)

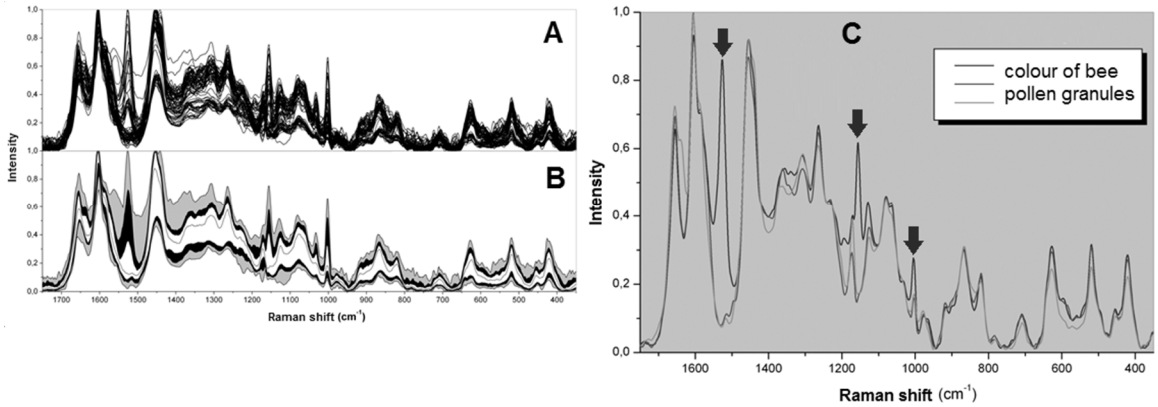

Fig. 7. FT Raman spectra (A) of all bee pollen granules *Papaver somniferum* (*n*=30) and statistical evaluation of the spectra (B); corresponding median spectra of the fractions (C); arrows indicate carotene bands

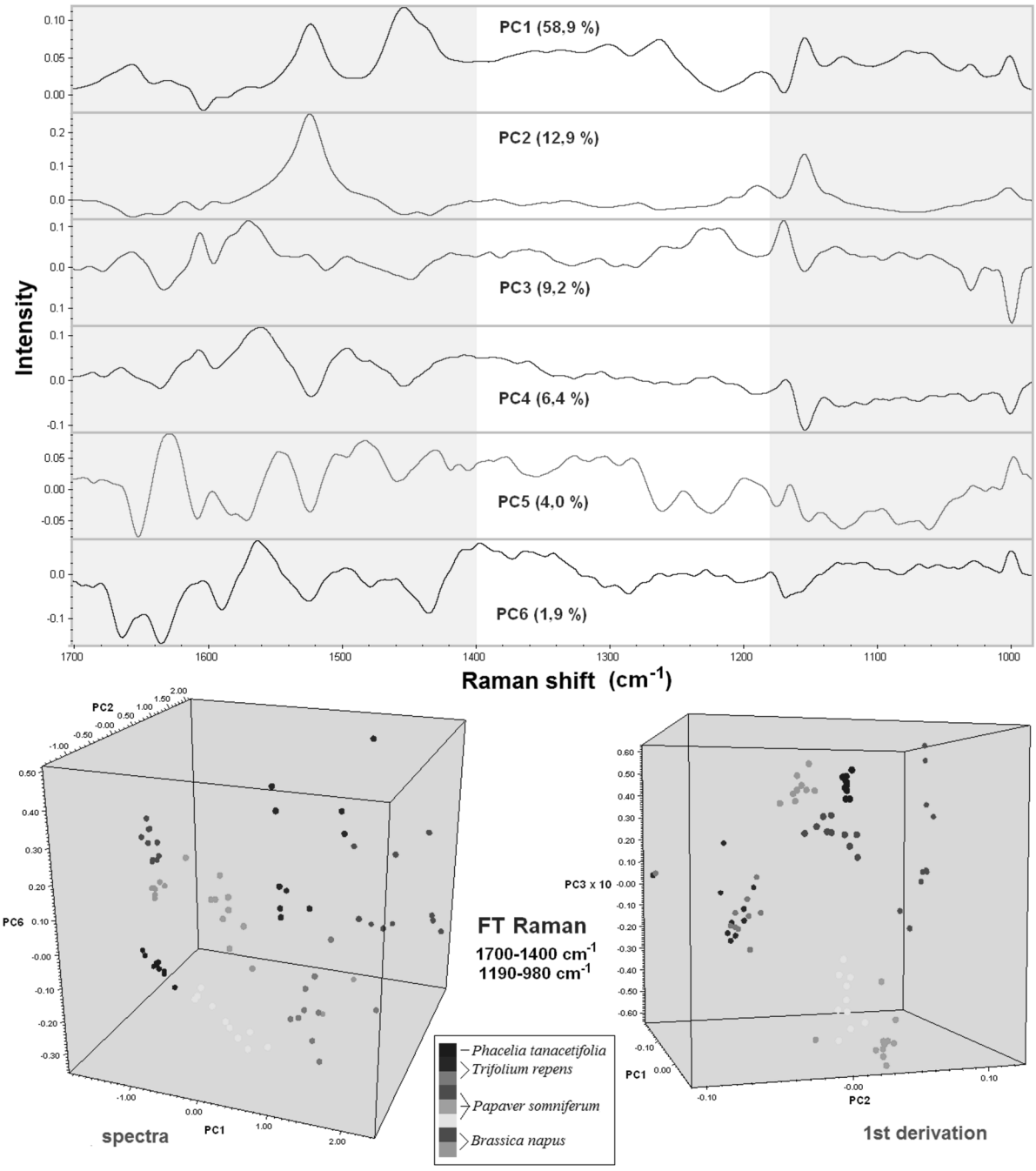

Fig. 8. Loading and 3D component score graphs for FT Raman spectra of bee pollen granules

Conclusions

Obtained results confirmed that vibration spectra of bee pollen granules are suitable for evaluation of bee pollen heterogeneity and detection of fractions originated from other botanical species. These methods are very sensitive to chemical composition of bee pollen. In addition, FT Raman spectra are able to detect differences in pigment composition that correlate with colour.

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ВИЗНАЧЕННЯ ПОХОДЖЕННЯ ПИЛКОВИХ ГРУДОЧОК ЗА ВІБРАЦІЄЮ СПЕКТРОСКОПІЧНИХ МАРКЕРІВ

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Анотація. *Робота присвячена визначенню розмаїття та розподілу пилкових грудочок у обніжжі чотирьох ботанічних видів рослин: Trifolium repens, Papaver somniferum, Brassica napus та Phacelia tanacetifolia. За допомогою ІЧ-Фур'є-спектроскопії в ближній (NIR) та середній (MIR) інфрачервоних областях, а також FT-Raman-спектроскопії комбінаційного розсіювання світла, проводили аналіз грудочок бджолиного пилку і встановлювали їх класифікацію відповідно до ботанічного походження.*

Отримані спектроскопічні дані статистично опрацьовані з використанням методу головних компонентів (PCA). Встановлено, що кожен з методів спектроскопії виявився придатним для класифікації бджолиного обніжжя на основі спектральних відмінностей. FT спектри комбінаційного розсіювання підтвердили, що наявність каротину істотно впливає на колір бджолиного обніжжя з маку.

Ключові слова*: спектроскопічний аналіз, бджолиний пилок, Trifolium repens, Papaver somniferum, Brassica napus, Phacelia tanacetifolia.*

ОПРЕДЕЛЕНИЕ ПРОИСХОЖДЕНИЯ ПЫЛЬЦЕВЫХ КОМОЧКОВ С ПОМОЩЬЮ ВИБРАЦИИ СПЕКТРОСКОПИЧЕСКИХ МАРКЕРОВ

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Аннотация. *Работа посвящена определению разнообразия и распределения пыльцевых комочков в обножке четырех ботанических видов растений: Trifolium repens, Papaver somniferum, Brassica napus и Phacelia tanacetifolia. С помощью ИК-Фурье-спектроскопии в ближней (NIR) и средний (MIR) инфракрасных областях, а также FT-Raman-спектроскопии комбинационного рассеяния света, проводили анализ комочков пыльцы и устанавливали их классификацию в соответствии с ботаническим происхождением.*

Полученные спектроскопические данные статистически обработаны с использованием метода главных компонентов (PCA). Установлено, что каждый из методов спектроскопии оказался пригоден для классификации пчелиной обножки на основе спектральных различий. FT спектры комбинационного рассеяния подтвердили, что наличие каротина существенно влияет на цвет пчелиной обножки из мака.

Ключевые слова: ***спектроскопический анализ, пчелиная пыльца, Trifolium repens, Papaver somniferum, Brassica napus, Phacelia tanacetifolia.***

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MORPHOLOGICAL CHARACTERISTICS OF COMMON BUCKWHEAT (*FAGOPYRUM ESCULENTUM* MOENCH) POLLEN GRAINS AND BEE POLLEN

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Annotation. *The aim of this study was to document the morphological characteristics of pollen grains and bee pollen of common buckwheat. Pollen*